

Plant Lesions Promote the Rapid Multiplication of *Escherichia coli* O157:H7 on Postharvest Lettuce[▽]

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Received 13 May 2008/Accepted 7 July 2008

Several outbreaks of *Escherichia coli* O157:H7 infections have been associated with minimally processed leafy vegetables in the United States. Harvesting and processing cause plant tissue damage. In order to assess the role of plant tissue damage in the contamination of leafy greens with *E. coli* O157:H7, the effect of mechanical, physiological, and plant disease-induced lesions on the growth of this pathogen on postharvest romaine lettuce was investigated. Within only 4 h after inoculation, the population sizes of *E. coli* O157:H7 increased 4.0-, 4.5-, and 11.0-fold on lettuce leaves that were mechanically bruised, cut into large pieces, and shredded into multiple pieces, respectively. During the same time, *E. coli* O157:H7 population sizes increased only twofold on leaves that were left intact after harvest. Also, the population size of *E. coli* O157:H7 was 27 times greater on young leaves affected by soft rot due to infection by *Erwinia chrysanthemi* than on healthy middle-aged leaves. Confocal microscopy revealed that leaf tip burn lesions, which are caused by a common physiological disorder of lettuce, harbored dense populations of *E. coli* O157:H7 cells both internally and externally. Investigation of the colonization of cut lettuce stems by *E. coli* O157:H7 showed that the pathogen grew 11-fold over 4 h of incubation after its inoculation onto the stems, from which large amounts of latex were released. The results of this study indicate that plant tissue damage of various types can promote significant multiplication of *E. coli* O157:H7 over a short time and suggest that harvesting and processing are critical control points in the prevention or reduction of *E. coli* O157:H7 contamination of lettuce.

Escherichia coli O157:H7 infections have been linked to fresh lettuce and spinach in the United States. Several of these outbreaks were associated with minimally processed and packaged quantities of these commodities (5–8, 11, 15–17). It remains unclear whether these outbreaks resulted from multiplication of the pathogen in the field before harvest, at the time of harvest, or during and after processing. Previous studies have revealed that *E. coli* O157:H7 has the ability to persist on leafy vegetables in the field (18) and to multiply in the phyllosphere of lettuce plants under warm and wet conditions in the laboratory (3). Cooley et al. (12) recently demonstrated that *E. coli* O157:H7 is prevalent in the watershed of a major lettuce and spinach production area in California. In addition, they reported the detection of the *E. coli* O157:H7 spinach outbreak strain in environmental samples taken at locations close to the spinach field linked to the outbreak. However, epidemiological investigations of the outbreaks that were linked to leafy greens in these areas failed to identify the exact source of the outbreak strains and the specific conditions that led to these epidemics (5, 8). Much remains unknown about the pre- and postharvest factors that may lead to an outbreak after a contamination event.

Harvesting and processing of lettuce inherently cause plant tissue damage. Frank and coworkers (22, 25) have demonstrated that *E. coli* O157:H7 attaches preferentially to the cut edges of lettuce leaves as well as to distinct features on the leaf

surface such as trichomes, stomata, and cracks in the cuticle. However, growth of *E. coli* O157:H7 after attachment to cut lettuce leaves at these preferential sites in comparison to that on intact leaves has not been quantified. Studies of modified-atmosphere packaging have shown that *E. coli* O157:H7 can multiply on cut lettuce over prolonged time periods during storage at temperatures ranging between 10 and 15°C (1), particularly when pretreated with warm chlorinated water (13, 20). Additionally, *Salmonella enterica* (9) and *Shigella sonnei* (27) grew more rapidly and to larger population sizes on chopped leaves of cilantro and parsley, respectively, than on whole leaves. In order to fully assess the contamination risk associated with minimally processed leafy greens, the comparative potential levels of growth of *E. coli* O157:H7 on intact leaves and on leaves damaged by various mechanisms at or after harvest need to be determined.

The objective of the present work was to study the role of various types of plant lesions caused mechanically or biologically, at or after harvest, in the multiplication of *E. coli* O157:H7 on lettuce. More specifically, the effect of plant damage caused by cutting the stem, by cutting or bruising the leaves, by physiological lesions such as tip burn, and by the soft-rot pathogen *Erwinia chrysanthemi* was investigated.

MATERIALS AND METHODS

Strains and culture conditions. A spontaneous rifampin-resistant mutant of *E. coli* O157:H7 strain H1827, a clinical isolate linked to an outbreak of infections associated with consumption of lettuce in Connecticut and Illinois in 1996 (17) and a gift from T. Barrett (U.S. Centers for Disease Control and Prevention), was used in this study and was described previously (3). For microscopy, strain H1827 was transformed with plasmid pGT-KAN, which harbors the gene encoding the green fluorescent protein (GFP) expressed from the kanamycin resistance gene promoter and which was described previously (4). This plasmid was

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[▽] Published ahead of print on 18 July 2008.



FIG. 1. Photograph of a stem disc cut from a mature romaine lettuce plant at harvest. The stem released a large quantity of latex (black arrow) from the laticifers upon wounding of the stem tissue. Stem discs such as this one were inoculated with *E. coli* O157:H7 and placed in a petri dish for incubation at 28°C and measurement of bacterial growth in the latex and cut surface.

stably maintained in strain H1827 and conferred to it intrinsic green fluorescence and gentamicin resistance. This GFP-labeled strain is referred to here as *E. coli* O157:H7 (pGT-KAN). To test the effect of soft rot on colonization of lettuce by H1827R, the human pathogen was coinoculated with a nalidixic acid-resistant strain of the plant pathogen *E. chrysanthemi* strain 3937 (19), a gift from Sylvie Reverchon (Université Lyon 1, France).

All strains were cultured to the early stationary phase of growth on a rotary shaker at 28°C in Luria-Bertani broth amended with rifampin (100 µg/ml) or nalidixic acid (50 µg/ml) and with gentamicin (15 µg/ml) as appropriate. For preparation of inocula, the cultures were washed twice by centrifugation in potassium phosphate buffer (KP buffer) (10 mM; pH 7) and resuspended in KP buffer (0.5 mM; pH 7) at the desired cell concentration based on absorbance at 600 nm.

Plant growth conditions. Romaine lettuce plants (*Lactuca sativa* cv. Parris Island) were used throughout these studies. The plants were grown to mature heads in Sunshine Mix 1 (Sun Gro Horticulture Distribution Inc., Bellevue, WA) in a greenhouse with a 16-h photoperiod and day and night temperatures of 24°C and 18°C, respectively, before the leaves were harvested for the experiments. The plants were fertilized weekly, starting at 2 weeks after emergence, with 1 mg of NKP (Spectrum Brands, Inc., Atlanta, GA) (20:20:20) per plant. Romaine lettuce heads that were grown and packed in boxes in the field were purchased directly from the distributor and used to test the effect of mechanical lesions on the growth of *E. coli* O157:H7.

Stem inoculations. The stem of mature heads of lettuce plants grown in the greenhouse was cut 2 cm above the soil line. A disc 1 cm in thickness was cut from the stem and placed on wet filter paper in a petri dish (Fig. 1). Each replicate disc came from a different plant. Three discs were placed per petri dish. A 50-µl drop of a 10⁴ cells/ml of *E. coli* O157:H7 suspension prepared as described above was spread in the latex drop oozing from the disc. The replicate petri dishes were covered, sealed with Parafilm M (American National Can, Chicago, IL), and incubated at 28°C.

Leaf inoculations. The inoculum suspension of 10⁴ cells/ml was prepared from *E. coli* O157:H7 cultures as described above. Green middle leaves that were part of the open rosette (ca. the 11th to 15th leaf in order of emergence) were harvested from mature lettuce plants and used to test the effect of plant damage on the growth of *E. coli* O157:H7. Each leaf was inoculated individually by holding it at its base and immersing it for 3 s in the bacterial suspension at up to 2 cm from the base to prevent the inoculum from penetrating the vascular tissue at the cut end of the leaf. The excess inoculum suspension was drained briefly

from the inverted leaves, and each leaf was treated in one of the four fashions described below.

In order to test the effect of different types of leaf injury on the growth of *E. coli* O157:H7, the inoculated leaves were left intact, mechanically bruised, cut into pieces, or shredded. Bruising was achieved by crushing the leaf blade by use of large tweezers at five locations transversally on each side of the main vein and once at the leaf tip; each bruise was ca. 2.5 cm in length and 0.5 cm in width. For cutting and shredding, each leaf was cut with a scalpel crosswise into 4-cm-wide and 1-cm-wide pieces, respectively. Leaves that were left whole served as control leaves. Each leaf or all of the pieces of one leaf were placed in one bag. The bags were then incubated at 28°C.

In order to study the effect of soft rot on the colonization of lettuce by *E. coli* O157:H7, 5-g samples of leaves cut crosswise into 4-cm-wide pieces were placed in a bag and coinoculated with 4 ml of a suspension containing cells of the plant pathogen *E. chrysanthemi* and of *E. coli* O157:H7 at 2×10^6 cells/ml of KP buffer (0.5 mM each). Two leaf age groups were tested: young leaves from the heart and leaves from the middle of the head (middle-aged leaves). Leaves of different age groups were placed in separate bags and incubated at 28°C.

For microscopic observation of *E. coli* O157:H7 in tip burn lesions, affected leaves were inoculated by immersing each inverted leaf in a suspension of *E. coli* O157:H7 (pGT-KAN) consisting of 10⁵ cells/ml of KP buffer (0.5 mM). The KP buffer and the lettuce plants were both maintained at 24°C for 4 h before inoculation was performed to prevent a temperature differential between the leaves and the inoculum suspension. Care was also taken not to immerse the area of the stem in order to avoid internalization of the inoculum cells in the stem. The leaves were then placed in a bag and incubated for 24 h at 28°C.

Measurement of bacterial populations on leaves and stems. For whole leaves that were bruised, cut, shredded, or left intact, each replicate sample consisted of one leaf or all pieces of one leaf incubated in a bag; for leaf pieces coinoculated with *E. coli* O157:H7 and *E. chrysanthemi*, each bag of 5 g of leaf pieces represented one replicate sample; and for stem discs, each replicate disc came from a different plant and was sampled from a different petri dish in which it had incubated. At each sampling time, KP buffer (10 mM) was added to each sample in a bag at the following volumes: for experiments testing the effect of mechanical plant damage, 100 ml; for those investigating soft rot, 50 ml; and for those investigating growth on stem discs, 10 ml.

The stem discs were sonicated in an Astramax Generator sonicator bath (Misonix Inc., Farmingdale, NY) at 250 W for 1 min and then rubbed vigorously by hand on all sides to remove the bacterial cells from the plant tissue. This procedure allowed for lower volumes of buffer to be used, thus lowering the bacterial detection limit for this small plant tissue sample. Inoculated leaves were processed in a Stomacher 400 system (Seward, West Sussex, United Kingdom) at high power for 2 min.

The resulting suspensions were plated with an automated plater (Autoplate 4000; Spiral Biotech Inc., Norwood, MA). Suspensions from leaves inoculated with *E. coli* O157:H7 were plated onto Luria-Bertani agar containing rifampin. Suspensions from leaves coinoculated with *E. coli* O157:H7 and *E. chrysanthemi* were plated onto Luria-Bertani agar containing rifampin and onto Luria-Bertani agar containing nalidixic acid for the measurements of the human and plant pathogen population sizes, respectively. Plates with *E. coli* O157:H7 were incubated at 37°C for 24 h, whereas plates with *E. chrysanthemi* were incubated at 30°C for 48 h. Population sizes on the leaves and stem discs were assessed by plate counts.

Microscopy. One-centimeter-thick leaf discs were sampled from tip burn lesions. The discs were mounted in AquaPoly/mount (Polysciences, Warrington, PA). The GFP signal from the bacteria and the red autofluorescence of the plant cells were visualized using a Leica TCS-NT confocal microscope (Leica Microsystems, Wetzlar, Germany) with emission filter sets BP525/50 and LP590, respectively.

Statistical analyses. All experiments were replicated at least twice. All statistical analyses were performed with Prism version 3.0 software (GraphPad Software, Inc., San Diego, CA).

RESULTS AND DISCUSSION

Growth of *E. coli* O157:H7 on cut lettuce stems. At harvest, lettuce plants are cut at the base of the stem. Therefore, the fate of *E. coli* O157:H7 on the cut stem after a potential contamination event at harvest was investigated in this study. Lettuce is one of few edible crops that produce latex (14, 23). Upon cutting of lettuce stems, a large quantity of latex is

TABLE 1. Growth of *E. coli* O157:H7 on the cut stems of romaine lettuce plants

Time of incubation (h)	Mean log (cells/disc) bacterial population size ^a	SEM	Fold population increase ^b
0	2.83A	0.03	
2	3.58B	0.03	5.62
4	3.87C	0.05	11.09
22	7.13D	0.11	20,090.93

^a Population size expressed as the mean of the log number of CFU/disc on four replicate stem discs. Mean values followed by different letters were significantly ($P < 0.05$) different by the Tukey-Kramer multiple comparison test.

^b Values represent increases in population size compared to that measured immediately after inoculation (0 h).

released from the laticifers onto the cut surface (Fig. 1). After harvest, the latex on the stems rapidly changes color from white to brown, dries, and can be transferred to other parts of the lettuce by rubbing of the stem of one head against the leaves of another. Browning and drying of the latex was also observed within the first hour after harvest in this study despite the incubation of the stem discs under humid conditions.

As early as 2 h and 4 h after its inoculation onto lettuce stem discs, population sizes of *E. coli* O157:H7 had increased 5.6- and 11.1-fold (Table 1). By 22 h of incubation, the *E. coli* O157:H7 population size on the stem discs had increased 20,091-fold, suggesting that the surface of cut lettuce stems holds large quantities of substrates that allow for the multiplication of *E. coli* O157:H7. In addition to the metabolites that leaked from the plant cells due to cutting, the presence of sugars in the lettuce latex (14) may have promoted the growth of *E. coli* O157:H7 on the cut stems. Indeed, after its inoculation into latex that was collected from lettuce stems and diluted 100-fold in distilled water, *E. coli* O157:H7 cells grew 10-fold within 12 h at 28°C (data not shown). Although lettuce latex contains letucenin A (23), a phytoalexin that has a role in the resistance of lettuce tissue to infection by bacterial plant pathogens such as *Pseudomonas syringae* pv. *phaseolicola* (2), this apparently did not prevent growth of *E. coli* O157:H7 on the cut stems.

Effect of mechanical lesions on the growth of *E. coli* O157:H7. During harvest and postharvest handling, lettuce leaves can be mechanically damaged by bruising or cutting. The effect of leaf damage on the growth of *E. coli* O157:H7 over a short period of time was tested in this study by (i) simulating crushing of the leaf blade with tweezers and (ii) generating various extents of leaf cuts by cutting the leaf into a few large pieces or into multiple narrow strips (shredding). Within only 4 h after inoculation and treatment, the *E. coli* O157:H7 population size increased 3.99-, 4.54-, and 11.05-fold on bruised, cut, and shredded leaves, respectively, but increased only 1.95-fold on intact leaves (Table 2). Whereas the *E. coli* O157:H7 population sizes on the leaves were all similar immediately after inoculation and treatment, they were significantly greater on cut and shredded leaves than on intact leaves after 4 h of incubation at 28°C (Tukey-Kramer test; $P < 0.05$) (Table 2). Shredding, which generated the highest proportion of damaged tissue per leaf, also promoted the largest *E. coli* O157:H7 populations, suggesting that colonization by the pathogen was correlated with extent of leaf damage. The rapid growth of *E.*

TABLE 2. Effect of various lettuce leaf preparation conditions on the growth of *E. coli* O157:H7 during 4 h of incubation of the leaves at 28°C

Leaf condition	Mean bacterial population size ^a		Fold population increase ^b
	0 h	4 h	
Whole	3.86A	4.15A	1.95
Bruised	3.63A	4.24A	3.99
Cut	3.86A	4.51B	4.54
Shredded	3.68A	4.72C	11.05

^a Population size expressed as the mean of the log number of CFU/g of leaf tissue on three and five replicate leaves at 0 and 4 h, respectively. Mean values followed by the same letter within the column were not significantly ($P < 0.05$) different by the Tukey-Kramer multiple comparison test.

^b Values represent increases in population size between 0 and 4 h after inoculation and treatment. For each leaf condition, the population size at 4 h was significantly ($P < 0.05$) different from that at 0 h, as revealed by the two-tailed unpaired *t* test.

coli O157:H7 on shredded leaves may have resulted from the enhanced attachment of *E. coli* O157:H7 cells to the broken plant tissue, as demonstrated previously (22, 24, 25), combined with higher availability of substrates to *E. coli* O157:H7 at those sites due to their leakage from the plant cells.

Lettuce is frequently stored in containers in the field for short periods of time during harvest operations until transported to the processing plant. Conditions of warm temperature and high humidity due to the wetting of the lettuce in order to preserve leaf turgidity may prevail during this time. The results of this study indicate that during only 4 h under the conditions described above, plant tissue damage can promote substantial growth of the pathogen and reduce the microbial safety of the product.

Role of soft rot and leaf age in *E. coli* O157:H7 colonization of lettuce. Plant pathogens can cause extensive postharvest damage of fresh produce. Soft rot is one of the most common postharvest diseases of lettuce. This disease increases the availability of nutrients to bacterial cells due to the enzymatic degradation of the plant cell wall by the plant pathogen and renders the leaf environment significantly more aqueous as cytoplasmic contents are released from the macerated cells.

The results of this study indicate that the population sizes of *E. coli* O157:H7 on lettuce were affected greatly by the presence of soft rot caused by coinoculation with the plant pathogen *E. chrysanthemi*. While *E. coli* O157:H7 continued to multiply over 44 h after coinoculation with *E. chrysanthemi*, its population declined on healthy leaves in the last period of incubation (Fig. 2A). The coinoculated leaves showed mild symptoms of tissue maceration typical of soft rot as early as 22 h postinoculation. At 44 h, the population sizes of *E. coli* O157:H7 were 3.3- and 6.2-fold greater on rotted leaves than on healthy leaves for young and middle-aged leaves, respectively (Tukey-Kramer test; $P < 0.001$). *E. coli* O157:H7 colonized the young lettuce leaves to a greater extent than was seen with the middle-aged leaves (Fig. 2A), as has been demonstrated previously (3). This leaf-age-dependent trend was not observed for the plant pathogen (Fig. 2B). Since leaf-age-dependent differences in bacterial population sizes on healthy leaves are dictated in part by differences in the nitrogen content of the exudates on their surfaces (3), it is likely that such

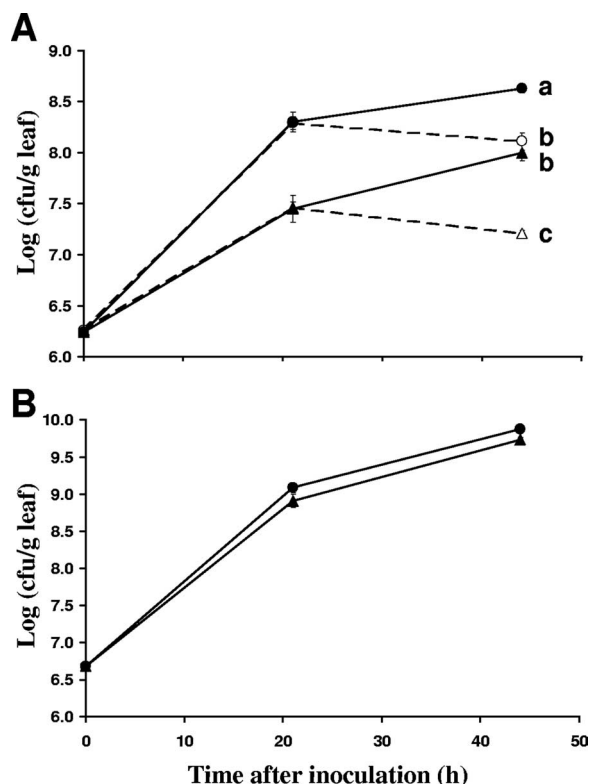


FIG. 2. Effect of soft rot and leaf age on the population dynamics of *E. coli* O157:H7 (A) and *E. chrysanthemi* (B). Comparative population sizes of *E. coli* O157:H7 on harvested healthy leaves inoculated with *E. coli* O157:H7 alone (dashed lines, open symbols) and on leaves coinoculated with *E. coli* O157:H7 and *E. chrysanthemi* are plotted; leaves developed visible symptoms of soft rot 22 h after inoculation (solid lines, closed symbols). The population sizes of *E. coli* O157:H7 (A) and *E. chrysanthemi* (B) on young inner (circles) and middle (triangles) leaves from the romaine lettuce head are shown. Each datum point represents the mean population size of *E. coli* O157:H7 on five replicate samples of cut leaf pieces. Bars represent standard errors of the means. Mean values followed by identical letters were not significantly ($P < 0.05$) different by the Tukey-Kramer multiple comparison test.

differences are overshadowed in the plant pathogen upon maceration of the plant tissue as a consequence of the resultant release of abundant nutrients.

It is noteworthy that the population size of *E. coli* O157:H7 on rotted young leaves was 27-fold greater than that on healthy middle-aged leaves, suggesting an additive effect of leaf age and soft rot. Therefore, young soft-rotted lettuce leaves represent an environment highly conducive to multiplication of *E. coli* O157:H7 on contaminated romaine lettuce.

In a survey of produce at the marketplace, Wells and Butterfield (26) observed an incidence of *Salmonella* species on fruit and vegetables affected by soft rot that was twice that seen on healthy produce. In laboratory studies, soft rot also had a positive effect on the population sizes of *Salmonella* spp. on potato, carrot, and bell pepper disks (26) and on that of *Listeria monocytogenes* on endive leaves (10). It is likely that the presence of aggregates or biofilms in the rotted tissue prevents the accurate estimate of the enteric pathogen population sizes by CFU counts and that these pathogens are thus even more

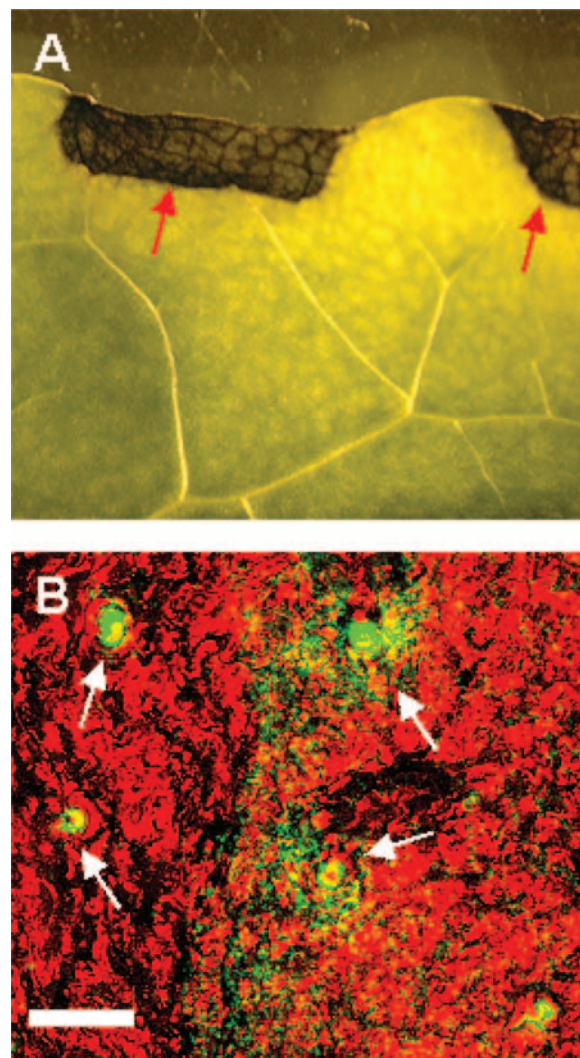


FIG. 3. Effect of tip burn lesions on colonization of lettuce by *E. coli* O157:H7. (A) Stereomicroscope image of tip burn lesions (red arrows) at the margin of a romaine lettuce leaf. (B) Confocal microscope image of *E. coli* O157:H7 (pGT-KAN) cells (green fluorescence) that colonized the necrotic tissue of a tip burn lesion shown in panel A during 24 h of incubation at 28°C. The pseudo-three-dimensional image was obtained by projection of a z series from the leaf surface into the leaf tissue. Large densities of *E. coli* O157:H7 cells are located in the stomatal openings (white arrows) and in the mesophyll layer. The necrotic and amorphous plant tissue is apparent due to the autofluorescence of the leaf in the red range. Yellow pixels were generated by overlay of green pixels from fluorescent bacterial cells and red pixels from the fluorescent plant tissue. Scale bar, 100 μ m.

numerous on diseased produce than was assessed. Therefore, the ubiquitous nature of soft-rot pathogens on leafy greens, and the considerable growth of enteric pathogens that they promote in macerated plant tissue, warrants the vigilance of the food industry and of consumers about symptoms of this disease on produce in order to prevent or minimize food-borne illness.

Internalization into tip burn lesions. A few leaves sampled from the plants grown in the greenhouse had typical tip burn lesions on the edge of the leaf blade (Fig. 3A). Tip burn is a

complex physiological disorder of lettuce that is characterized by necrosis of the leaf margins (21) and is common in many lettuce production areas of the United States. As evidenced by the dark polygonal outline of the necrotized cells in the tip burn lesion shown in Fig. 3A, the plant cells appear to keep their shape during necrosis. Therefore, this type of plant damage is different from soft-rot disease in which the plant cells are macerated. Examination of tip burn lesions under the confocal microscope revealed the presence of *E. coli* O157:H7 cells at high densities not only on the surface of the plant cells but also inside the dead tissue and stomata (Fig. 3B). These high densities of internalized cells suggest that tip burn lesions are conducive to multiplication of ingressed *E. coli* O157:H7 cells, where they may be protected on lettuce leaves from adverse conditions in the pre- and postharvest environment.

Overall, the studies described herein revealed that mechanical damage, and biotic and abiotic diseases, of lettuce can greatly enhance its colonization by *E. coli* O157:H7 after harvest. The ability of *E. coli* O157:H7 bacteria to increase in numbers on the cut leaves and stems of lettuce as much as 11-fold within only 4 h is important to take into account in the development of new good agricultural practices and hazard analysis and critical control point protocols. Additionally, the presence of lesions on the leaves often leads to soft rot, a postharvest disease that was shown to further enhance the growth of *E. coli* O157:H7 on lettuce in this study. In view of the numerous outbreaks of *E. coli* O157:H7 infections linked to minimally processed leafy greens and of the low infectious dose of *E. coli* O157:H7 in humans, such opportunities for *E. coli* O157:H7 to multiply in hospitable niches on postharvest lettuce in a short time should be considered in risk assessment studies. They also emphasize the critical need to avoid preharvest contamination at any level, since even a small number of pathogen cells could yield minimal infectious doses if postharvest amplification resulting from any of the scenarios described herein were to occur.

ACKNOWLEDGMENTS

Thanks are given to Jennifer Kyle, Danielle Goudeau, and Aileen Haxo for technical assistance and to Robert Mandrell for review of the manuscript.

This work was supported by a grant from the U.S.-Israel Binational Agricultural Research and Development Fund and by funds from the U.S. Department of Agriculture, Agriculture Research Service (CRIS project 5325-42000-044-00D).

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